Pergamon

0031-9422(95)00272-3

Phytochemistry, Vol. 40, No. 4, pp. 1065–1070, 1995

0031-9422/95 $9.50 + 0.00

LIPIDS AND FATTY ACIDS IN GUARD-CELL PROTOPLASTS FROM *VICIA FABA* LEAVES

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(*Received* 10 *January* 1995)

Key Word Index—*Vicia faba*; Leguminosae; broad bean; guard-cell protoplasts; mesophyll-cell protoplasts; polar lipids; neutral lipids; fatty acids.

Abstract—Guard-cell and mesophyll-cell protoplasts were isolated from *Vicia faba* leaves, and their lipids and fatty acids quantitatively analysed. All the glycerolipids and fatty acids that occurred in mesophyll-cell protoplasts were found in guard-cell protoplasts. On a total fatty acid basis, levels of chloroplast lipids (monogalactosyldiacylglycerol, digalactosyldiacylglycerol and sulphoquinovosyldiacylglycerol) and phosphatidylglycerol were lower and their constituent fatty acids were more saturated in guard-cell protoplasts than in mesophyll-cell ones. In contrast, levels of extrachloroplastic lipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and cardiolipin) were higher in guard-cell protoplasts than in mesophyll-cell protoplasts. These results suggest that extrachloroplastic membrane systems, including mitochondria, are more developed than chloroplast membranes. In addition, guard-cell protoplasts contained a large amount of triacylglycerols, a probable component of spherosomes, lipid droplets and/or plastoglobuli, which are frequently observed in guard cells.

INTRODUCTION

Stomatal aperture, which is controlled by volume changes in guard cells, regulates gas-exchange between a plant and the environment. Stomatal opening which corresponds to the swelling of guard cells, is induced by the accumulation of K^+, its closing corresponding to shrinking of the cells, being caused by release of K^+. To understand the mechanisms of volume regulation in guard cells, many studies have been carried out on the pumps and channels in the plasma membranes and on the activities of photosynthesis and respiration that provide energy for this ion transport [1–3].

It is clear that the physiological and biochemical properties of guard cells are quite different from those of mesophyll cells. Guard cells have a higher activity of respiration than their mesophyll counterparts on a volume basis; respiration provides the main energy source required for stomatal movement [4, 5]. Furthermore, guard cells develop signal transduction systems and can respond to various kinds of environmental stimuli, such as light, growth regulators (e.g. abscisic acid and auxin), air pollutants, CO_2 and Ca^{2+} [1, 3].

Despite extensive investigations of the physiology and biochemistry on guard cells, there are only a few reports on the chemical analysis of their cellular components. Recently, protein profiles of guard-cell protoplasts subjected to SDS-PAGE, which differs largely from that of mesophyll-cell protoplasts, have been shown [6]. With respect to the lipid profile of guard cells, Sato [7] has shown lipid biosynthesis from acetate in guard-cell protoplasts from *Vicia*; however, he failed to quantify each lipid class because of the low yields obtained from the protoplasts.

In order to achieve a better understanding of the structural and functional characteristics of guard cells, we have determined the content and composition of the fatty acids and glycerolipids in guard-cell and mesophyll-cell protoplasts isolated from leaves of *Vicia faba*.

RESULTS AND DISCUSSION

Evaluation of guard-cell and mesophyll-cell protoplasts

Guard-cell protoplasts were isolated from *Vicia* epidermis peeled from 80–120 leaves. The isolated protoplasts yielded $5–9 \times 10^6$ cells in each experiment. Microscopic observations showed that no mesophyll cells and less than 1% of epidermal cells were found in the protoplast preparations on the basis of cell number (Fig. 1). Mesophyll-cell protoplasts were isolated from 10–20 leaves of *Vicia*. There was no contamination in the protoplast preparations by other cell-types (Fig. 1).

Isolated guard-cell and mesophyll-cell protoplasts had high activities of photosynthetic O_2 evolution with the

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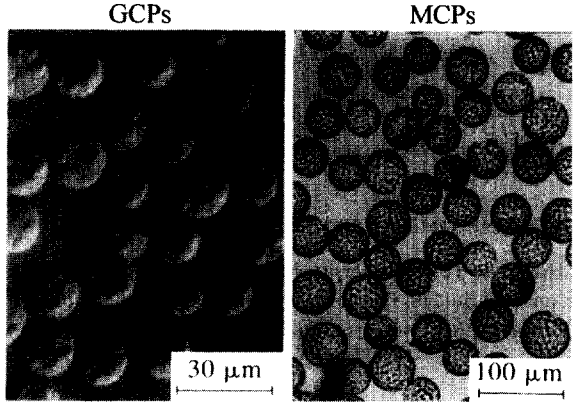

Fig. 1. Photomicrographs of guard-cell protoplasts (GCPs) and mesophyll-cell protoplasts (MCPs) isolated from *Vicia faba* leaves. Guard-cell protoplasts were observed under a differential interference microscope. Note the difference in size between the protoplasts.

rates of 200–250 and 100–150 μmol mg^{-1} chlorophyll hr^{-1}, respectively, under red light illumination. Proton pumping was elicited in guard-cell protoplasts by a pulse of blue light (200 μmol m^{-2} sec^{-1} for 30 sec) under the background red light (800 μmol m^{-2} sec^{-1}) and their magnitudes were *ca* 0.3 nmol μg^{-1} protein pulse^{-1}. These values are close to those in activities and magnitudes as previously reported in these cell-types from *Vicia* [8–10].

Content of fatty acids and protein in guard-cell protoplasts

The content of total fatty acids in guard-cell protoplasts was found to be one-quarter of that in mesophyll-cell protoplasts on a cell basis (Table 1). Since guard-cell protoplasts from *Vicia* have a much smaller volume (*ca* 1/20) and a much lower content of chlorophyll (*ca* 1/80) than mesophyll-cell protoplasts on a cell basis [4], the content of total fatty acids is calculated to be richer in guard-cell protoplasts than in mesophyll-cell protoplasts on both volume and chlorophyll bases. This suggests that guard cells have more dense intracellular membranes and/or richer storage lipids than mesophyll cells on a volume basis.

The content of protein in guard-cell protoplasts was *ca* one-sixth that of mesophyll-cell protoplasts on a cell basis (Table 1); the concentration of protein in guard cells was also higher than their mesophyll counterparts.

Composition of fatty acids in guard-cell protoplasts

Among the peaks detected in the gas chromatogram of the fatty acid methyl esters obtained from guard-cell protoplasts, palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), α-linolenic acid (18:3), arachidic acid (20:0), icosenoic acid (20:1), icosadienoic acid (20:2), icosatrienoic acid (20:3), behenic acid (22:0) and lignoceric acid (24:0) were identified by comparison with R_t on GC and the patterns of mass spectra of the standard esters. We also identified two other peaks as hexadecenoic acids (16:1) from their mass spectra, that were further confirmed to be 3-*trans*-16:1 and 9-*cis*-16:1 by comparison with the corresponding authentic standards. Furthermore, the total ion chromatogram from GC-MS showed the occurrence of docosenoic acid (22:1), albeit in trace amounts, in guard-cell protoplasts.

Table 2 presents the molar composition of fatty acids in the lipid extracts from guard-cell and mesophyll-cell protoplasts. Two differences were found. Firstly, C_{16} and C_{18} fatty acids were more saturated in guard-cell protoplasts than in mesophyll-cell ones. The amount of 18:3, which is abundant in chloroplast galactolipids of mesophyll cells [11], was particularly low in guard-cell protoplasts, but was mainly compensated for by increases in the amounts of 18:1 and 18:2. Secondly, the contents of long-chain fatty acids, C_{20}, C_{22} and C_{24} were higher in guard-cell protoplasts. This is consistent with the active biosynthesis of 20:0 and 22:0 from acetate in guard-cell protoplasts, as well as in epidermal-cell ones [7]. These fatty acids are probably involved in the synthesis of cuticular wax components [12].

Table 1. Content of fatty acids and protein in guard-cell and mesophyll-cell protoplasts from *Vicia faba* leaves

	Guard-cell protoplasts	Mesophyll-cell protoplasts	Whole leaves
Volume (ml 10^{-9} cells)*	2.14	49.0	—
Chl (mg 10^{-9} cells)*	2.0	158	—
Protein (mg 10^{-9} cells)	220	1390	—
Total fatty acids (μmol 10^{-9} cells)	440	1760	—
Total fatty acids (μmol ml^{-1})	206	35.9	—
Total fatty acids (μmol mg^{-1} Chl)	220	11.1	11.1
Total fatty acids (μmol mg^{-1} protein)	2.0	1.3	0.77

*Data from Ref. [4].

Content of total fatty acids represents the sum of 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 22:0 and 24:0 in lipid extracts. Values are averages of results from 4–7 separate samples in each case. Chl, chlorophyll.

Table 2. Fatty acid composition in total lipid extracts of guard-cell and mesophyll-cell protoplasts from *Vicia faba* leaves

Samples	Fatty acid composition (mol %)											
	16:0	16:1*	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	24:0
GCPs	15.6	1.3	5.6	11.7	31.1	29.9	1.5	1.5	0.5	0.3	0.8	0.3
MCPs	8.2	4.5	1.6	4.5	9.6	70.9	0.3	0.2	< 0.1	< 0.1	0.2	< 0.1
Whole leaves	9.3	4.1	1.7	6.0	13.7	63.9	0.4	0.3	0.1	< 0.1	0.3	< 0.1

*Sum of 3-*trans*-16:1 and 9-*cis*-16:1 (see text).

From GC-MS measurements, we also identified 22:1 in total lipids from guard-cell protoplasts (less than 0.1%). Values are averages of results from 5–8 separate samples in each case. GCPs, guard-cell protoplasts; MCPs, mesophyll-cell protoplasts.

Chloroplast lipids in guard-cell protoplasts

Figure 2 shows the content of polar and neutral lipids in guard-cell and mesophyll-cell protoplasts; it is expressed on a total fatty acid basis (Table 1) in order to compare the distribution of lipids. All the glycerolipids that generally occur in green leaves [11] were detected in both types of protoplasts, though their contents differed. The amounts of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulphoquinovosyldiacylglycerol (SQDG), specific to chloroplast membranes, were lower in guard-cell protoplasts than in mesophyll-cell ones. The content of phosphatidylglycerol (PG), which is associated mainly with chloroplasts [13], was also lower in guard-cell protoplasts. Since the content of total fatty acids was lower in guard-cell protoplasts than in their mesophyll counterparts on a cell basis (Table 1), these results suggest that guard cells contain fewer numbers of chloroplasts and/or poorer development of thylakoids. Electron microscopic observations confirm these morphological characteristics of guard cells [14]. Higher degrees of fatty acid saturation in these lipid classes (Table 3) might be related to poorly developed thylakoids. On a volume basis, however, the content of chloroplast lipids is calculated to be similar or rather higher in guard-cell protoplasts than in mesophyll-cell ones (*cf.* Table 1 and Fig. 2A).

Extrachloroplastic lipids in guard-cell protoplasts

In contrast to the chloroplast lipids, contents of the extrachloroplastic lipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and cardiolipin (CL), were found to be higher in guard-cell protoplasts than in mesophyll-cell ones (Fig. 2A). This suggests that guard cells contain extrachloroplastic organelle(s) in larger number and/or with more highly developed membrane systems. The higher amount of CL is noteworthy (Fig. 2A). Bligny and Douce [15] have reported that CL is restricted to inner membranes of mitochondria in cultured sycamore cells. This is also probably the case with *Vicia* guard cells, since morphological observations show the occurrence of numerous mitochondria in guard cells to be a common feature in various plant species, including *Vicia* [14]. In addition,

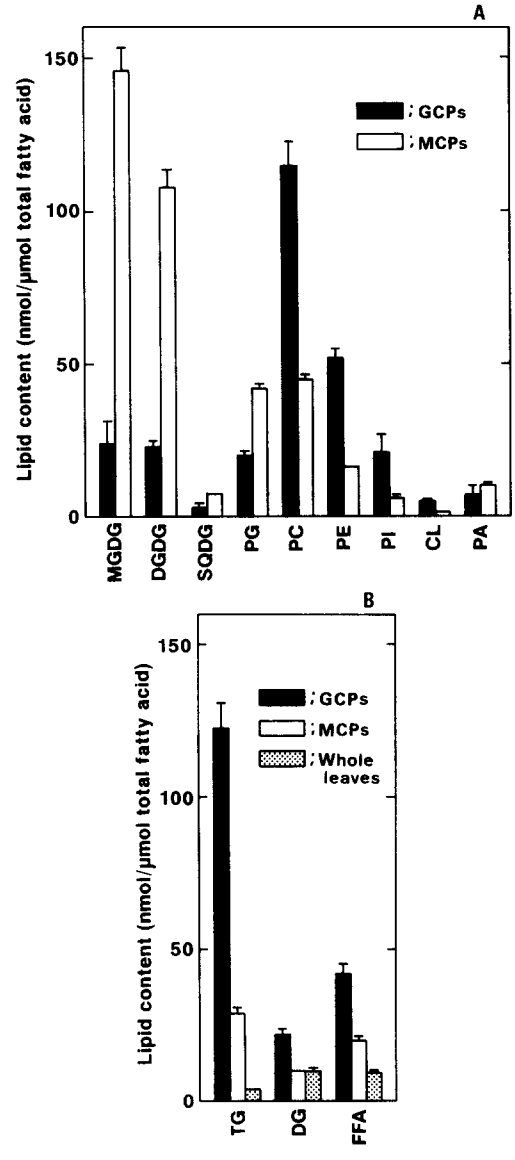

Fig. 2. Content of individual (A) polar lipids, and (B) neutral lipids in guard-cell protoplasts (GCPs) and mesophyll-cell protoplasts (MCPs) isolated from *Vicia faba* leaves. Mean value ± s.d. of results from three separate samples is given in each case. PA, phosphatidic acid; DG, sum of 1,2-diacylglycerol and 1,3-diacylglycerol. See text for other abbreviations.

Table 3. Fatty acid composition of major glycerolipids in guard-cell and mesophyll-cell protoplasts from *Vicia faba* leaves

Lipids	Samples	Fatty acid composition (mol %)											
		16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	24:0
MGDG	GCPs	2.1	1.2	1.0	5.2	11.2	79.4	0	0	0	0	0	0
	MCPs	1.4	0.4	0.3	0.9	2.1	95.0	0	0	0	0	0	0
DGDG	GCPs	7.9	1.2	5.2	2.7	6.5	76.5	0	0	0	0	0	0
	MCPs	9.4	tr	1.3	1.0	1.4	86.8	0	0	0	0	0	0
PG	GCPs	42.8	10.1*	3.2	11.0	19.1	13.3	0.2	0	0	0	0.1	0.2
	MCPs	14.4	41.4*	1.4	4.3	8.8	29.7	tr	0	0	0	0	0
PC	GCPs	18.0	tr	5.3	22.1	32.0	18.5	1.5	1.4	0.3	0.2	0.5	0.2
	MCPs	18.8	tr	3.3	21.9	25.1	28.2	0.8	0.6	0.2	0.2	0.4	0.5
PE	GCPs	22.2	tr	5.0	16.9	36.4	12.1	1.2	2.9	1.0	0.7	1.0	0.6
	MCPs	24.3	1.6	3.7	17.2	32.3	17.0	1.1	1.1	0.6	0.4	0.5	0.2
TG	GCPs	16.3	3.1	3.5	16.1	29.7	26.8	1.7	1.0	0.3	0.2	1.1	0.4
	MCPs	11.6	4.8	2.3	10.3	17.8	52.1	0.2	0.3	0.5	0.1	0	0.1
	Whole leaves	20.6	7.8	5.5	15.9	24.8	23.5	0.8	0.4	0	0	0.3	0.4

*3-*trans*-16:1.

GCPs, guard-cell protoplasts; MCPs, mesophyll-cell protoplasts; tr, trace (less than 0.1%).

the high respiratory activities of *Vicia* guard-cell protoplasts have been demonstrated, viz, a high rate of KCN-sensitive O_2 uptake and high activity of cytochrome oxidase [4]. Portions of PC, PE and PI may participate in the membrane constituents of the mitochondria.

As shown in Table 3, fatty acid species and their composition of PC and PE in guard-cell protoplasts were comparable to those in mesophyll-cell ones. However, we note that amounts of long-chain fatty acids (C_{20}, C_{22} and C_{24}) were higher in these phospholipids of guard-cell protoplasts; these acids were rarely detected in the chloroplast lipids of both protoplasts (Table 3). Abdul-Karim *et al.* [16] have shown that acetate is actively incorporated into long-chain fatty acids of PC and neutral lipids in leek epidermal cells. Murata *et al.* [17], however, have shown that these acids are restricted to phosphatidylserine in various organs of 18 plant species. Thus, fatty acid analysis of phosphatidylserine will provide more detailed distribution of long-chain saturated and unsaturated fatty acids in guard cells; we have confirmed the occurrence of this phospholipid in guard-cell as well as mesophyll-cell protoplasts (results not shown).

Recent investigations have shown that exogenous inositol triphosphate induces Ca^{2+} release in the cytoplasm of guard cells from *Commelina*, triggering the closing of stomata [18, 19], and that 1,2-diacylglycerol (1,2-DG) activates the plasma membrane H^+ pump, leading to stomatal opening [20]. If these compounds take part in the signal transduction of intact leaves as second messengers, polyphosphoinositides should occur and be actively turned over in guard cells by the analogy with those in animal cells [21]. As shown in Fig. 2A, we found a considerable amount of PI in guard-cell protoplasts from *Vicia*. However, we could not detect phosphatidylinositol mono- or bisphosphate (both lipids to be less than 0.5 nmol μmol^{-1} total fatty acid) on TLC plates using sprays of primuline, H_2SO_4 (both for general lipid detection) or molybdenum blue (phospholipid detection). This is probably due to the limitations of the sensitivity of our analytical techniques; radiotracer experiments will be needed for more precise determination of these minor but important phospholipids.

Neutral lipids in guard-cell protoplasts

Guard-cell and mesophyll-cell protoplasts were found to contain larger amounts of triacylglycerol (TG) than whole leaves (Fig. 2B). Increases in TG in mesophyll-cell protoplasts are probably due to the enhanced production of TG during the isolation procedure [22]. We have recently proposed that TG is produced in leaf cells under various stresses as a result of the enzymatic conversion from MGDG via 1,2-DG and free fatty acid (FFA), with concomitant production of oligogalactolipids, such as tri- and tetragalactosyldiacylglycerol (TGDG and TTGDG) [23, 24]. Since the procedure for protoplast isolation appears to be a strong stress factor for plant cells, we could expect TG in mesophyll-cell protoplasts to be produced via this pathway [23]. As shown in Table 4, TGDG and TTGDG did accumulate in mesophyll-cell protoplasts but not in whole leaves, demonstrating the operation of the pathway from MGDG to TG during isolation of the protoplasts.

In contrast, we failed to find these oligogalactolipids in guard-cell protoplasts (Table 4). This suggests that TG is the original lipid constituent in guard cells. Since spherosomes, lipid droplets and plastoglobuli are often observed in guard cells [14, 25], TG may at least be partly located in these organelles.

Since TG generally function as an energy reserve in plant and animal cells, it is likely that TG in guard cells is utilized for a substrate of mitochondrial respiration through β-oxidation. In accord with this, guard-cell protoplasts survive much longer than mesophyll-cell proto-

Table 4. Relationships between contents of triacylglycerols (TG) and oligogalactolipids in guard-cell protoplasts, mesophyll-cell protoplasts and whole leaves of *Vicia faba*

Samples	Lipid content (nmol μmol^{-1} total fatty acid)				
	TG	MGDG	DGDG	TGDG	TTGDG
Guard-cell protoplasts*	94.5	27.3	25.0	nd	nd
Mesophyll-cell protoplasts*	26.7	82.3	60.7	2.4	2.7
Whole leaves	2.7	87.1	57.0	nd	nd

*Guard-cell protoplasts had blue light-dependent H^+-pumping activity of 0.29 nmol μg^{-1} protein pulse^{-1}. Mesophyll-cell protoplasts had photosynthetic O_2-evolving activity of *ca* 100 μmol mg^{-1} chlorophyll hr^{-1}.

Guard- and mesophyll-cell protoplasts were isolated from the same leaves. Values are averages of results from two separate samples in each case. nd, not detected (less than 0.2 nmol μmol^{-1} total fatty acid).

plasts from the same plant species in the dark [26]. Recent investigations indicate that guard-cell chloroplasts have an extended longevity in yellow and senescent leaves, even when mesophyll chloroplasts are degraded completely, and that stomata have a capacity to open and close in these leaves [27]. Thus, it is possible that TG acts as an effective energy source for respiration and provides energy for stomatal movement in senescing leaves.

EXPERIMENTAL

Plant materials and protoplast preparation. Plants of *Vicia faba* were grown hydroponically from seeds in a greenhouse for 4–8 weeks [10]. Guard-cell and mesophyll-cell protoplasts were enzymatically isolated from the abaxial epidermis of 80–120 leaves [10] and the remaining 10–20 leaves devoid of epidermis [8], respectively. Isolated guard-cell and mesophyll-cell protoplasts were washed twice with 0.4 M mannitol containing 1 mM $CaCl_2$ and 0.6 M mannitol containing 1 mM $CaCl_2$, respectively. Protoplasts were finally suspended in 0.5 ml of the same composition of washing medium.

Lipid extraction. Immediately after protoplast prepn, their suspensions (0.4 ml) were mixed with 4 ml of boiling *iso*-PrOH, followed by boiling the mixt. until most of the *iso*-PrOH had evapd. Whole leaves of *Vicia faba* were similarly treated with boiling *iso*-PrOH. Total lipids were extracted from the boiled residues according to Ref. [28]. To examine the occurrence of polyphosphoinositides, total lipids were extracted from guard-cell protoplasts with $CHCl_3$–MeOH–3.4 N aq. HCl (6:6:1) according to Ref. [29], followed by the partitioning in aq. and organic phases [28]. The organic phase containing polyphosphoinositides was recovered, concd under N_2, dissolved in 5 ml of $CHCl_3$ containing 0.05% (w/v) butylated hydroxytoluene as an antioxidant and stored at − 20° until use [30].

Separation and quantification of lipids. Neutral lipids were sepd by TLC on silica gel developed with hexane–Et_2O–HOAc (70:30:1). Polar lipids, except for oligogalactolipids and polyphosphoinositides, were sepd by 2D TLC on silica gel using $CHCl_3$–MeOH–H_2O (65:25:4) in the first and $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O (10:4:2:2:1) in the second dimension; $CHCl_3$–MeOH–conc. NH_3-*iso*propylamine (13:7:1:0.1) in the second dimension was also used. Oligogalactolipids (TGDG and TTGDG) were sepd according to Ref. [23], and polyphosphoinositides were sepd by TLC on potassium oxalate-impregnated silica gel as described in Ref. [31]. Spots were visualized and identified according to Ref. [30]. In some expts, spots were charred by spraying with 50% H_2SO_4 and heating at 150°. Fatty acid Me esters from the individual lipids and the total lipid exts were prepd according to Ref. [30] with 15:0 as int. standard and quantified by FID-GC [32]. When the Me ester frs were contaminated with pigments, they were purified by TLC before the analysis by GC [32].

To identify fatty acid species in the total lipids of guard-cell protoplasts by GC-MS, fatty acid Me esters were chromatographed on a fused silica capillary column (60 m × 0.25 mm i.d., SP-2330; Supelco) with He as carrier; the column temp. was prog. at 175–215° for 2° min^{-1}. MS were recorded at an ionization voltage of 70 eV.

Photosynthetic and proton-pumping activities. Rates of photosynthetic O_2 evolution in guard-cell and mesophyll-cell protoplasts were determined according to Refs [4] and [8], respectively. Proton-pumping activities in guard-cell protoplasts were measured as described in Ref. [10].

Content of chlorophyll and protein. Chlorophyll was extracted with 80% aq. Me_2CO and determined spectrophotometrically [33]. Protein was measured according to Ref. [34] with BSA as standard.

Acknowledgements—The authors thank Dr M. Yamada (Hokkaido Tokai University) for valuable discussions during the course of the work.

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